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SYMPOSIUM

ON

SOIL MICRO-ORGANISMS AND PLANT WELL-BEING

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SYMPOSIUM ON SOIL MICRO-ORGANISMS AND PLANT WELL-BEING

Held at the 20th Annual Meeting of the Indian Academy of Sciences—Belgaum December 1954

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ABSTRACTS

The modern views on *in situ* production of antibiotics in soils and their uptake by plants and the role of heavy metals in the production of these toxins, the derangement in the uptake of metabolites by plants and its repurcussions on amino acid sequence of fungal wilt polypeptide toxins are discussed by Sadasivan. The more recent work on root exudations in plants and their import on rhizosphere microfloras is presented.

Subramanian discusses the ecological and taxonomic problems in the genus *Fusarium*.

Some aspects of the synecology of soil fungi are discussed by Ramakrishnan. He also reviews the techniques evolved for the study of soil fungi with special reference to the "dilution plate" and "root burial" techniques.

Certain aspects of variation and variability in the genus *Fusarium*, formation of variants by different means, and how these affect the taxonomy of the genus are discussed by Venkata Ram.

The *in vivo* reactions of cotton plants infected by *F. vasinfectum* as indicated by ascorbic acid and carbohydrate metabolism as well as the factors affecting the *in vitro* action of the pure toxin, fusaric acid, produced by this pathogen are discussed by Kalyanasundaram. The antibiotic potency of this phytotoxin, its production and role in soil are also discussed.

Lakshminarayanan reviews the theories of fusariose wilts of plants with special reference to N_2 distribution and derangement in cotton wilt. He also discusses the chromatographic survey of nitrogenous constituents in the plants and the possible role of iron and cystine in the mechanism of resistance.

The indispensability of heavy metals in fungal nutrition is discussed by Saraswathi Devi, stressing the care and experimental precautions involved in bringing out their essential nature. The effects of certain heavy metal deficiencies on growth and metabolism of some vascular wilt pathogens (*Fusarium* spp.) are given.

Lakshmanan discusses the respiratory changes in cotton plants (resistant and susceptible strains) with reference to *Fusarium* wilt of cotton. Respiratory changes brought about by toxins and growth substances are also discussed.

IT gives me great pleasure to be here and to be called upon to open this Symposium. I extend my warmest thanks to Prof. Sir C. V. Raman for this invitation and I feel certain that my colleagues who are to follow this brief survey also feel honoured to be here to-day.

There seems little doubt that soils all the world over are a dynamic medium for micro-organismal growth and activity. In recent years much of the fundamental attack on hitherto obscure phenomena connected with enzyme chemistry in so far as it relates to micro-organisms of the soil has been partly, if not wholly, resolved and we are, as it were, in a more fortunate position when we could discuss the role of micro-organisms in plant well-being. With the discovery of antibiotics and their production, *in situ* by soil microbes and their subsequent movement into plants as detected in their guttation fluid by using new bioassay techniques (Brian, Wright, Stubbs and Way, 1951), there has been considerable speculation and an increased curiosity to understand not only the path of these *in situ* and *in vivo* toxins through the root systems to the metabolically active shoots but also *vice versa* of sprayed materials on leaf surfaces. In short, from the search for systemic fungicides by using what appeared at first to be a fundamental discovery of limited importance, *viz.*, the bioassay of these fungicides in the guttation fluids of plants, has emerged considerable volume of applied work of great promise. It is relevant to point out that these systemic fungicides seem to interfere with key enzyme systems and produce derangement of key metabolites and it is getting increasingly evident that substances important for fungal metabolism if inactivated, the chain of derangement starts. Indeed, inside the plant intense chemical changes take place and the recent astounding progress in the field of heavy metals and tissue response, particularly, in the cases of involvement of Fe, Cu and Mg in enzyme systems of plant tissues places this field of enquiry in the forefront. The role of Mn and, to a lesser extent, Zn in assuming a key position inasmuch as twenty enzyme systems have been shown to be activated by Mn alone, particularly, in general enzymatic decarboxylation and hydrolysis and in group transfer has posed new and interesting theoretical assumptions. Further, our own work on vitamin requirements of fungi heterotrophic to vitamins (Sadasivan and Subramanian, 1954; Apparao, Saraswathi-Devi and Suryanarayanan, 1955) indicates that in the presence of the vitamin of choice there is an inter-dependence on heavy metals. I have to cite also the case of the indispensability of Zn for fusaric acid production by *Fusarium vasinfectum* details

of which will be given later by Miss Saraswathi-Devi. It is hardly necessary for me to emphasise here that the *Aspergillus niger* technique for the detection of very minute traces of some of the heavy metals as used by many workers needs no special commendation as every serious worker in this field would have realised by now the value and elegance of this technique in conjunction with chromatography in dealing with metals of the order of one gamma or less. This will be dealt with by Miss Saraswathi-Devi and Mr. Lakshminarayanan. Apart from these studies, which are intrinsically important in understanding the physiology of the host, the fungal worker tackling problems of toxæmia in plants from soil-borne infections has not kept pace with plant virus work in the study of biochemical changes in plant tissues in a pathological condition. For instance, Stanley (1943) postulated from his analytical results on the amino acid set up in tobacco mosaic virus that " the presence of histidine in the rib-grass strain and its absence from tobacco mosaic virus demonstrates conclusively that the mutation of a virus may be accompanied by the introduction of an entirely new amino acid and the conversion of tobacco mosaic virus into the rib-grass strain must have been accompanied by the introduction of several hundred histidine molecules into the virus structure". This has been vigorously commented upon by Bawden (1950): " Such statements postulate as facts events about which there is no evidence whatsoever, for nothing is known of the origin of the rib-grass strain, although *a priori* it is perhaps unlikely that it arose as a mutation from tobacco mosaic virus. The most that can reasonably be assumed is that they all derive from a common ancestor, but for how long the different strains have been following different evolutionary courses, and how many separate mutations have led to their present conditions, cannot be guessed even approximately. Hence, mutation and differences between virus strains would seem at least as likely to result from changes in sequence of amino acids on a peptide chain as, particularly, the terminal amino acids is important in determining specificity as shown in polypeptides." It is not my intention to draw any close parallel between this and the fungal wilt problem especially when dealing with the strainal question in such pathogens. Nevertheless, it seems to me that as many of the fungal wilt toxins recently isolated are polypeptides there may be need to explore the possibility of critically studying the amino acid sequence in them. It is also equally clear that the enzyme systems of these organisms and their strains in the presence of heavy metals *in vitro* and *in vivo* have to be critically studied as it may throw considerable light on the physiological states of the resistant and susceptible varieties of hosts in nature.

I now wish to turn my attention to a related problem of *in vivo* toxæmia of fungal wilts of plants. I am referring to the question of the rhizosphere where there is an intense biological activity resulting in microbial antagonisms and interaction between the micro-organisms and plant roots resulting in a severe competition for the available metabolites. Our work in this field (Agnihothrudu, 1954) indicates a significant increase in microbial numbers in the rhizosphere region with advance in age of cotton plants, the full import of which observation is not clear. Again, fungal and bacterial numbers were greater in the rhizosphere of susceptible cotton plants as compared with the resistant ones. The limitations in arriving at more than tentative conclusions in these observations has been the difficulty of determining the nature of exudates that are likely to become available in the rhizosphere region to the competing micro-organisms and the nature of the tissue response by the hosts to the presence of the increased microfloral populations in that region. Some progress in this field has been made by us in recent months by subjecting root exudations to chromatographic analysis but our tentative findings are not presented here for lack of confirmation. However, mention must be made of a very recent publication (Katznelson, Rouatt and Payne, 1954) where striking increase was observed in amino acids when leach water from roots of plants from dried sand were remoistened. The leach water showed chromatographically a variety of amino acids including glutamine and aspartic acids, leucine, alanine, cysteine, glycine, lysine, phenylalanine and proline. Apart from the chromatographic detection of these amino acids, biologically speaking amino acid requiring bacteria gave confirmatory evidence by making better growth in this leach water.

The basic problem, therefore, is to appreciate the complexity of root exudations in relation to microbial growth and activity and to devise methods by which qualitative and quantitative data could be obtained to enable us to go beyond the region of speculation and understand the implications of root exudations in relation to microbial activity in the rhizosphere region. I see no reason why apart from the exudation of amino acids, there could also not be exudations of heavy metals which may be selectively absorbed, translocated and exuded depending on the optimum or sub-optimum metabolic status of the wilting host and also absorption and exudation of B-vitamins necessary for the growth of vitamin heterotrophic fungi. In the data to be presented by my colleagues today there are several experimental evidences obtained in our laboratory on the many facets of experimental reasoning in this field of specialisation. I do not propose to add more to the clime of this Symposium and would like to conclude by stating that much effort could well be put in to remedy the inadequate experimental techniques

now in the hands of the microbiologist who deals with the complicated problem of host-parasite physiology without which a close understanding of the intricacies of nature may well be impossible.

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THE ECOLOGICAL AND THE TAXONOMIC PROBLEMS IN FUSARIA*

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The small contribution which I wish to make to this Symposium will deal with two aspects of the study of Fusaria in which I have been interested for some years past, *viz.*, the ecology and the taxonomy of Fusaria, especially those which occur in soils.

The Ecological Problem in Fusaria

Much of the work on the ecological problem in the Fusaria has been aimed at elucidating the possible method of perennation of several soil-borne Fusaria. The work of Sadasivan (1939) on *Fusarium culmorum* gave an impetus to investigations along the same lines on vascular wilt Fusaria and it was shown that *F. vasinfectum*, the cotton wilt pathogen, like *F. culmorum*, possessed competitive saprophytic ability and could be classified as a soil-inhabitant (Subramanian, 1946). Further work on the occurrence of Fusaria in black cotton and other soils from southern India indicates that several other species of *Fusarium* can also be placed in the soil-inhabitant group. This list would include *F. chlamydosporum* Wr. and Rg., *F. avenaceum* (Fr.) Sacc., *F. equiseti* (Corda) Sacc., *F. scirpi* Lamb. and Fautr., *F. scirpi* Lamb. and Fautr. v. *acuminatum* (Ell. and Ev.) Wr., F. *scirpi* Lamb. and Fautr. v. *caudatum* Wr., *F. oxysporum* Schlecht., *F. javanicum* Koord., *F. javanicum* Koord. v. *radicicola* Wr., *F. solani* (Mart.) App. and Wr., *F. solani* (Mart.) App. and Wr. v. *martii* (App. and Wr.) Wr., *F. solani* (Mart.) App. and Wr. v. *minus* Wr., *F. solani* (Mart.) App. and Wr. v. *striatum* (Sherb.) Wr. and *F. cæruleum* (Lib.) Sacc. Stover's (1953) work indicates that *F. oxysporum* Schlecht. v. *cubense* (Smith) Wr. and Rg., which causes the panama disease of bananas exhibits a similar behaviour.

Whilst the soil-inhabitant type of behaviour appears to be the most common in this genus, a contrasting type of behaviour is shown by *F. udum* Butler which causes a vascular wilt of *Cajanus cajan* in India. That this species is distinct from *F. oxysporum* Schlecht. emend. Snyder and Hansen has been stressed by Subramanian (1955). *F. udum* has been shown

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(Agnihothrudu, 1954) to be incapable of colonising dead plant debris in soils and its saprophytic survival is limited to tissues originally invaded during its pathogenic phase—a behaviour similar to that of *Ophiobolus graminis*.

Work on saprophytic behaviour of soil Fusaria is obviously important from the point of view of control of the diseases caused by them and future work should be aimed at screening as many Fusaria as possible for their saprophytic behaviour in soils.

The Taxonomic Problem

One of the major difficulties encountered in all work with Fusaria is, indeed, their taxonomy. Notwithstanding the earlier work of Appel, Wollenweber, Reinking, Sherbakoff and others (see Wollenweber and Reinking, 1935) and the more recent studies of Snyder and Hansen (1940, 1941, 1945, 1954), the taxonomic problem of the Fusaria still remains. Wollenweber and Reinking's (1935) is, indeed, the most outstanding and authoritative treatise on this difficult subject and Wollenweber's monographic treatment (Wollenweber, 1931, 1943) brings together in an admirable manner exhaustive descriptions of the various taxa and also lists of synonymy, hosts, exsiccatæ and distribution. Snyder and Hansen's revision of the genus is based essentially on the broader groupings of the genus proposed by Wollenweber and Reinking, and alterations are effected only with reference to the species concept. Both systems have their protagonists and it is not necessary here to dwell at length on the comparative merits and demerits of the two systems (for which please see Padwick, 1941). On the other hand, it would be worthwhile discussing possibilities of a newer approach to the solution of this old problem.

A common feature of the Wollenweber-Reinking and the Snyder-Hansen classifications and also the more recent revision by Gordon (1952, 1954) is the implicit faith laid in the fundamentals of the classification of the genus as enunciated nearly thirty years ago by Wollenweber Sherbakoff, Reinking, Johann and Bailey (1925), a pre-requisite for taxonomic study of any given taxon being a pure culture derived from a single spore. It is necessary to grow such a single spore culture on several culture media and record their cultural characters at different stages of growth and also study the morphology before it could be placed in any known species. The procedure involved in specific identification is thus lengthy and complicated. Such a procedure would certainly be worthwhile if there is some hope of arriving at a correct specific identification. Unfortunately, this is seldom the case and the species concept of Wollenweber and Reinking is based on a mixture of morphological and cultural characters, of which the latter are highly variable and hence

are of little taxonomic value. The Snyder-Hansen system and also Gordon's revision both suffer from the same defects since the main modification effected is the reduction in the number of species on the basis of observations on the variability in cultures of the various taxa.

A different approach to *Fusarium* taxonomy was offered by the recent work of Miller (1945, 1946 *a*, 1946 *b*) in Canada who indicated the need to base the classification of the genus on a study of the 'wild type' in the various species. Miller's 'wild type' refers to a pure culture obtained immediately after isolation from the substratum. On the basis of a study of first cultures of a few isolates of Elegans Fusaria obtained from diseased muskmelon plants in Canada, Miller concluded that the 'wild type' in the genus *Fusarium* is the type that usually "occurs in nature as a form (or forms) that, when cultured on most artificial media, produces abundant aerial mycelium on which conidia, mostly non-septate, are borne rather sparsely" (Miller, 1945, p. 41). However, continued culturing, according to Miller, results in the loss of the 'wild type', being crowded out by mutants which are characterised by prolific sporulation (0–3- or more-septate conidia) and an adpressed type of growth with little or no aerial mycelium. Following up this hypothesis, Miller (1946 *a*, 1946 *b*) has further suggested that the traditional sporodochium in the genus *Fusarium* is synonymous with patch mutants encountered during his studies and, hence, has suggested that these should not be the basis for a sound classification of the genus. In my studies of first cultures of various parasitic Fusaria obtained from different genera of host plants and of saprophytic forms obtained from soils, no confirmation could be obtained of Miller's concept of a stereotyped and uniform 'wild type' (Subramanian, 1951). First cultures of both pathogenic and saprophytic Fusaria were not characterised by any uniform 'wild type', but fell into several intergrading types from the typically sporodochial or pionnotal to the typically mycelial. The Fusaria studied included *F. cæruleum* (Lib.) Sacc., *F. conglutinans* Wr. v. *citrinum* Wr., *F. culmorum* (W. G. Sm.) Sacc., *F. equiseti* (Corda) Sacc., *F. javanicum* Koord. and its variety *radicicola* Wr., *F. oxysporum* Schlecht., *F. scirpi* Lamb. and Fautr. and its varieties *acuminatum* (Ell. and Ev.) Wr., and *longipes* (Wr. and Rg.) Wr., *F. semitectum* Berk. and Rav., *F. solani* (Mart.) App. and Wr., and its varieties, *martii* (App. and Wr.) Wr., *minus* Wr., and *striatum* (Sherb.) Wr., and *F. vasinfectum* Atk. In a later investigation on several isolates of Fusaria obtained from corms of gladiolus plants showing yellows at Cambridge (England) during 1950, I found that these Fusaria exhibited a wide range of variation in cultural characters like amount of aerial mycelium colouration on different media, presence or absence of sporodochia and,

pionnotes, etc., but the morphological features of the conidia were remarkably constant. More recently, a similar wide range of variation in cultural characters has been reported in the case of first cultures of *F. udum* also (Subramanian, 1955). Here again the morphology of the micro- and macroconidia in the first cultures showed little variation compared to the cultural characters. Studies of this kind on ' wild types ' or first cultures thus point to the fact that a given species may exhibit a wide range of variation in cultural characters but not in morphological characters relating to the conidia. Nevertheless, the ' wild types', as defined by Miller, are not of universal application in the genus *Fusarium* since they do not conform to any single definite pattern and since in the definition suggested by Miller the emphasis has been on cultural characters.

In my opinion, the solution of the problem warrants an entirely new approach. It would appear that too much emphasis has been laid in the past on artificial cultures of organisms in taxonomic studies and firm adherence to the idea that, for taxonomic purposes, organisms should be studied under strictly controlled nutritional levels and other conditions in the laboratory is not always essential. In the eagerness to obtain cultures, the natural material is seldom examined and more often than not a non-sporulating culture has been obtained by continued subculturing of the fungus in synthetic media. The fungi have then to be fed on special concocted foods to obtain a culture in *normkultur* or *hochkultur*. In the case of several vascular wilt Fusaria, the pathogen is confined essentially to the vascular tissues of the host during the earlier stages of pathogenesis, but gradually invades the outer tissues progressively with the death of the infected plants and, indeed, sporodochia and numerous conidia are produced only during the final phase of the disease, following death of plant parts. Thus, the *hochkultur* stage in most of these specialised vascular wilt Fusaria appears on host tissues killed by the fungus but not earlier, and attempts to obtain, on artificial media, the *hockhultur* of the fungus from invaded tissues during earlier phases of the disease have very often failed. The case of Fusaria which cause foot rots and root rots appears to be different in that the invasion of the host tissues is more general during early phases of the disease and the conidia are produced earlier too. In any case, the conidia, in all species of Fusaria, are produced in the infected tissues or substrata sooner or later and such conidia as occur on tissues invaded in nature should be considered normal for all taxonomic purposes. There is also the question of variation among pure cultures of Fusaria and the limitations of working with such pure cultures would be realised by all those who have keenly followed the work of Hansen (1938) on the dual phenomenon in the Fungi Imperfecti.

All this circumlocutory procedure can be avoided by studying Fusaria as they occur in nature. Systematic work and taxonomic studies on various other genera of the Fungi Imperfecti are being carried out with precision without the aid of artificial cultures and solely on specimens collected directly from the field and occurring on the natural substratum. Such a study of Fusaria as they occur in nature would obviate all limitations resulting from the use of artificial cultures; and, as for the possible criticism that, in nature, spores of more than one species may occur side by side, thereby vitiating specific determination and taxonomic groupings, it may be pointed out that no two spores which are morphologically indistinguishable, having, of course, regard to their origin and dispersal, need be considered as taxonomically different. Another possible objection to the procedure recommended here would be the view that the taxonomic picture obtained by examining material procured directly from nature would be vitiated by the fact that conditions prevailing in nature may not be uniform and that a specific diagnosis drawn up out of such material may not be comparable with that of a specimen of the same species collected under different conditions of habitat, etc. This difficulty can be overcome by including in studies of this type a representative collection of specimens from different localities, etc. No taxonomic study would be perfect without a comprehensive investigation of a number of individuals of a given taxon obtained from different areas, different substrata, etc. What is important is the range of variation in key characteristics of a taxon as it occurs in nature rather than under laboratory-made artificial cultures. Cultures certainly have a major place in the study of the biology, physiology and various other aspects of molds, but their importance in taxonomy has been overemphasised. *Hochkultur* or *normkultur* cultures, if readily available, could certainly be used for taxonomic purposes; but the point I wish to emphasise here is that such cultures are not usually available, especially if the cultures had been maintained on rich synthetic media over a long period and in such cases the material obtained directly from nature provides the complete taxonomic picture. That appears to me the easiest and the most straightforward and simple approach to the solution of the problem and hence well worthy of serious consideration. Under the taxonomic procedure suggested here, the various species of Fusaria would be what they are in nature and not what they appear to be in culture.

My own findings are that it would be possible to identify Fusaria as they occur in their natural habitats without the aid of artificial cultures on synthetic or natural media; but here again a pre-requisite would be the reclassification of the genus on the basis of investigation of natural material and type and other exsiccatæ of the various taxa. This raises the funda-

mental question of the characteristics of the genus which are of value taxonomically. The genus was established by Link in 1809. It is usually placed in the Moniliales-Tuberculariaceæ-Mucedineæ-Phragmosporæ and includes by description hyphomycetes with septate, branched, hyaline vegetative hyphæ and producing hyaline conidia typically in sporodochia, the sporodochia being considered the distinguishing characteristic of the Tuberculariaceæ. Three asexual spore forms are recognised, *viz.*, the micro-conidia, the macro-conidia and the chlamydospores. The conidia, in general, are slime spores and are borne typically on phialides. That the sporodochium is a hallmark of the genus is shown by the occurrence of sporodochia in a number of species of this genus as they occur in nature, but less frequently on culture media. However, in artificial cultures many forms have been reported to occur which lack sporodochia, but otherwise agreeing with the genus in other characteristics. Such non-sporodochial forms (*e.g.*, *Fusarium poæ* (Pk.) Wr., *F. chlamydosporum* Wr. and Rg., *F. semitectum* Berk. and Rav., *F. camptoceras* Wr. and Rg., *F. bostrycoides* Wr. and Rg., *F. conglutinans* Wr., *F. orthoceras* App. and Wr., and *F. angustum* Sherb.) are also included within the genus by Wollenweber and Reinking (1935) and by Snyder and Hansen (1940, 1941, 1945). Indeed, the *raison d'etre* of this classification appears to be the same as the one recently stressed by Hughes (1953, p. 578), *viz.*, " that the species whose conidiophores are solitary, in tufts on a small stroma or in synnemata, are all congeneric." Apart from the application of this conclusion to the Fungi Imperfecti as a whole, its application to the genus *Fusarium* appears logical and should have approval, unless it be that future studies on natural material reveal the consistent occurrence of sporodochia in all species of the genus.

What then is the hallmark of the genus *Fusarium*? To my mind, it would be the typical hyaline, falcate or sickle-shaped phragmo-phialospores produced by the fungus. The classification of the genus should be based primarily on the nature of these phialospores, *i.e.*, their shape, septation and size. Some other characteristics, also recorded from specimens obtained directly from nature, like the nature of the conidiophores, the manner of conidial formation, the occurrence of smaller or micro-conidia and their morphology are also important. Most other characteristics like the presence or absence of chlamydospores and all cultural characters would appear to be of no value in taxonomy. Pathogenicity has been used as a criterion to classify species, varieties and forms by Wollenweber and Reinking and to classify forms by Snyder and Hansen. There is considerable evidence pointing to the fact that pathogenicity is a highly variable factor and it has been shown that several Fusaria have a wider host range than suspected. For instance,

F. oxysporum Schlecht. emend. Snyder and Hansen f. *vasinfecta* (Atk.) Snyder and Hansen is now known to be pathogenic to plants other than cotton, and the classification of the Elegans and other sections by Snyder and Hansen on the basis of selective pathogenicity has little experimental evidence to support it when applied to the genus as a whole. In such cases it would be superfluous and fantastic to accord any taxonomic status to the forms on the basis of their pathogenicity to one or more hosts. On the other hand, these forms could be given a biologic status within the genus since what they exhibit is biologic specialisation. The main argument in favour of this point of view is that biologic characters are highly variable especially in the case of facultative parasites and hence their use in taxonomy should be discouraged. A further criticism which would apply here is that the conditions optimum for pathogenicity are themselves ill-defined and variable so that it would be unwise to found a classification on such a criterion.

A thorough study, on these lines, of Fusaria as they occur in nature, collected from geographically different areas and from diverse substrata coupled with an enquiry towards an understanding of type specimens and other exsiccatæ, wherever possible, would pave the way for a taxonomic revision of the genus which would at once be easier to use and more in keeping with the characteristics of its individuals as they occur in nature.

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SOME ASPECTS OF SOIL FUNGAL ECOLOGY*

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Nearly forty years ago Waksman (1916 *a*, 1916 *b*, 1917) demonstrated by his pioneering work that the soil which was till then considered an inert medium, was really a dynamic ecological habitat teeming with an active fungal population. Since then numerous other workers all over the world have investigated the fungal floras of various kinds of soils. Most of the literature on the fungi isolated from soils has been brought together by Gilman (1945). Many facts of synecological significance have emerged from these floristic studies; but these studies by themselves are not strictly ecological. The recent studies of Zachariah (1949), Warcup (1951) and Tresner, Backus and Curtis (1954), however, are exceptions. Many autoecological studies have also been undertaken by plant pathologists in their attempts to unravel the life processes of plant pathogenic fungi. As a result of such studies, soil-borne plant pathogenic fungi have been broadly classified as ' root inhabitants ' and ' soil inhabitants ' (Garrett, 1950, 1951, 1952). On the other hand, facts regarding the influence of factors of the environment on the life of the large mass of soil fungi which are not known to be pathogenic and hence are not of immediate economic value, have to be gleaned from the numerous floristic studies referred to earlier. These findings are briefly discussed below with reference to the author's own work on the synecology of the fungi of three soils in the neighbourhood of Madras.

Techniques

Many techniques have been devised for the quantitative and qualitative study of soil fungi. These could be broadly grouped as (1) direct isolation from soil by plating methods, and (2) indirect isolation from previously colonised organic matter or artificial media. The ' dilution plate ' technique of Waksman (1922) as modified by Brierley, Jewson and Brierley (1928) is a bacteriological technique, adapted for soil fungal studies. This technique has been largely used for quantitative and qualitative studies. Many workers have pointed out its limitations both for counting the numbers of fungi and for their isolation. Modifications have been suggested by Warcup (1950) who, instead of plating out soil dilutions, plated measured quantities of soil

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and demonstrated that fungi belonging to the Hymenomycetes could be made to appear in his 'soil plates'. Jensen's (1935) modification of the Rossi-Cholodny slide technique and the method described by Jones and Mollison (1949) have been claimed to give more accurate pictures of the relative abundance of fungal mycelium in the soil. These, however, because of the difficulty of operation, have not come into general use, and Waksman's technique still remains the most widely used. The technique of indirect isolation from colonized organic matter or artificial media can generally be called the 'baiting' technique, as, in all cases suitable baits are offered to the fungi for colonization. Butler (1907) was the first to use this technique, using hemp seeds as baits, for studying soil and aquatic Phycomycetes. Many later workers on the Phycomycetes like Sparrow (1943) have extensively and successfully used this technique. The 'straw burial' technique of Sadasivan (1939) as modified by Subramanian (1946) has been used in this laboratory for the study of soil-borne Fusaria with special reference to their occurrence and survival in soils. Zachariah's (1953) work has further helped to standardise this technique. It may be relevant to refer here to an experiment conducted by the author (Ramakrishnan, 1953) to assess the comparative merits of the 'root burial' and 'dilution plate' techniques. Fungi, as shown in the following table (Table I) were isolated by these two techniques from three soils at Vandalur, near Madras.

It is clear that neither of the techniques brought out all the fungi present in the soil. Fungi appearing on the dilution plate belonged mostly to the dry-spored group (Fungi imperfecti and Mucorales) producing enormous numbers of rather thick-walled, minute spores. These fungi are mostly saprophytes and are probably capable of withstanding microbial antagonism. These fungi probably exist in the free soil as spores most of the time. Fungi appearing on the rot bits on the other hand included strong cellulose decomposers like *Memnoniella echinata*, *Chætomium* spp. and *Fusarium* spp. It is also interesting to note that such 'soil inhabiting' plant pathogens like *Neocosmospora vasinfecta*, *Macrophomina phaseoli* and *Fusarium solani* appeared only on the root bits. Similar results have been reported by Zachariah (1953) also. It seems reasonable to assume that these fungi do not exist in the free soil, but inside vegetable debris which they colonise, and remain there either as mycelium or in some resting stage. The finding of Venkatram (1952) that *Fusarium solani* could be induced to form abundant chlamydospores by bacterial action is of special interest in this context.

Chinnayya's (1952) ingenious adaptation of the root burial technique by the use of ammonia to isolate *Coprinus* spp. indicates that this technique has not been fully exploited, and much remains to be done in this field.

TABLE I

Fungi isolated from Vandalur Soils

Fungi appearing on dilution plates	Fungi appearing on root bits	Fungi common to both techniques
Cunninghamella echinulata Thaxter	*Pythium mamillatum* Meurs	*Cunninghamella bertholletiæ* Stadel
Arthrobotrys sp.	*Syncephalis cornu* Van Tiegh. & LeMonnier	*Mucor racemosus* Fres.
Aspergillus sydowii (B. & S.) Thom & Church	*Syncephalis reflexa* Van Tiegh.	*Thielavia basicola* Zopf.
Aspergillus terreus Thom	*Chætomium trilaterale* Chivers	*Aspergillus niger* Van Tiegh.
Aspergillus ustus (Bainier) Thom & Church	*Chætomium* sp.	*Aspergillus nidulans* (Eidam) Winter
Aspergillus variecolor (B. & Br.) Thom and Raper	*Neocosmospora vasinfecta* Smith	
Penicillium sp.	*Helminthosporium microsorum* P. Henn.	
Cladosporium herbarum (Pers.) Link	*Helminthosporium nodulosum* B. & C.	
Curvularia lunata (Wakker) Boed.	*Memnoniella echinata* (Riv.) Galloway	
Curvularia maculans (Bancroft) Boed.	*Fusarium solani* (Mart.) App. & Wr.	
Curvularia pallescens Boed.	*Fusarium solani* (Mart.) App. & Wr. var. *minus* Wr.	
Hormodendrum sp.	*Macrophomina phaseoli* (Maubl.) Ashby	
Septonema indica Ramakr. sp. nov.*		

* To be described elsewhere.

Baiting techniques where artificial media are substituted for organic substrata have been described by Chesters (1948) and Evans (1954). The former demonstrated that his perforated ' immersion tubes ' filled with nutrient agar could be used for isolating some rapidly growing fungi. The soil ' recolonization tube ' has been used by Evans to study the rate of recolonization of sterilized soil by fungi.

An interesting technique has been described by Campbell (1951) for isolating *Phytophthora cinnamomi* Rands from infested soils. Here, holes bored in fresh apples are packed with infested soils when the fungus grew into the tissue of the fruit and then could be easily isolated in pure culture. The use of live indicator plants to demonstrate the presence of obligate parasites like *Plasmodiophora brassicæ* Wor. has been described by Macfarlane (1952). In this laboratory, *Pythium aphanidermatum* (Eds.) Fitzpatrick has been consistently isolated from garden soil by a thick sowing of mustard seeds. Given optimum conditions of moisture the seedlings were invariably damped off and got covered with a pure growth of the fungus.

Ecology

From techniques we may pass on to a brief consideration of the chief factors which influence the abundance and distribution of soil fungi. It is generally agreed that these factors are moisture, organic matter, reaction of the soil and the major nutrients, nitrate nitrogen, phosphorus and potassium and certain heavy metals.

Most investigators have found a direct correlation between moisture content and numbers of fungi (Waksman, 1944; Dixon, 1929 and Jasevoli, 1924). Cobb's (1932) work has shown that this correlation may not be clearly seen to the same extent in all soils. The author (Ramakrishnan, 1953) also found that moisture content and fungal numbers were directly correlated in the soil of a rice field soil at Vandalur near Madras while in two other soils no such clear correlation was observable. However, the soil with the highest moisture-holding capacity had consistently higher numbers of fungi than those with lower moisture-holding capacities. It is, perhaps, not logical to look for any unqualified correlation between any factor and fungal numbers in such a complex medium as the soil where so many conflicting influences operate.

It is but natural that organic matter should have a great influence on the numbers of fungi in the soil. The investigations of Waksman (1916 *a*, 1916 *b*, 1917 and 1944), Ma (1933) and Jensen (1935) confirm this view. The author's studies on Vandalur soils also agree with this conclusion to a large extent.

Regarding the effec. of the three major constituents of plant food, *viz.*, nitrogen, phosphorus and potassium, as far as the author is aware there is no report in literature where the changes in these constituents in *natural soil* were correlated with their fungal numbers. There are, however, numerous reports on the effect of mineral amendments on the behaviour of soil fungi. It has been found that nitrate nitrogen, phosphorus and potassium stimulate the development of soil fungi (Waksman and Starkey, 1924). The author (Ramakrishnan, *loc. cit.*) found that in Vandalur soils the variation in nitrate nitrogen and phosphorus were directly correlated with numbers of fungi, while the effect of potassium was not clear, possibly because this factor did not appreciably vary from month to month.

Although no report on the influence of heavy metals on the general fungus flora of the soil is available, the work done in this laboratory (Sadasivan, 1951) on the influence of boron, zinc and manganese on the colonization and survival of Fusaria in soils, demonstrates that this aspect need further study.

Finally the influence of plant cover on the microflora of the soil should be mentioned. Recent studies on the rhizosphere microfloras of various plants (Katznelson, Lochhead and Timonin, 1948; Agnihothrudu, 1953; Chinnayya and Agnihothrudu, 1953) emphasize the importance of such studies in understanding the ecology of soil fungi. The work of Tresner *et al.* (1954) is also of great interest here.

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CERTAIN ASPECTS OF TOXICOLOGICAL STUDIES WITH SPECIAL REFERENCE TO *FUSARIUM VASINFECTUM* ATK.*

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Most of the workers on toxicological studies in the genus *Fusarium* have based their conclusions primarily upon the production of toxins by these pathogens in synthetic media and *in vitro* studies of these toxins. Recent evidence of *in vivo* experiments done in this laboratory (Kalyanasundaram, 1953 and 1954; Subba Rao, 1954) largely seems to support the conclusions of *in vitro* studies done here (Kalyanasundaram, 1953) and elsewhere (Gäumann, Naef-Roth and Kobel, 1952). A detailed study of the symptomatology of cotton plants infected by *Fusarium vasinfectum* (Kalyanasundaram, 1954; Subba Rao, 1954), as well as biochemical studies on these infected host plants (Kalyanasundaram, 1953) has led us to the logical conclusion that wilting is more due to chemical agents than mere physical causes. This will be considered briefly here before entering into other aspects of this toxicological problem.

The indispensability of ascorbic acid in plant metabolism and its role as a phytohormone is well-known (Virtanen, 1936). In *Fusarium vasinfectum* infected cotton plants, between the period of infection in the root region and symptom appearance in the aerial parts—this period was found to vary from 5 to 8 days—the ascorbic acid content was very much above the normal. This period, when there was no visual toxic symptom (Satyanarayana and Kalyanasundaram, 1952), could be called the period of incipient toxæmia as Hageman, Hodge and McHargue (1942) have indicated the possibility of using ascorbic acid measurements in plants as an index of the health of the plants. Both in this stage and in the subsequent stage of the disease, when the first visual symptom had made its appearance on the host, there was inhibition of starch synthesis along the regions of vein clearing, coupled with lowered ascorbic acid metabolism. There was, however, considerable transport of synthesised food materials from the leaves to the root region (Kalyanasundaram, 1954) until the necrosis of the stem set in and the plant collapsed. In fact, Waggoner and Dimond (1952) have shown in tomato

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plants infected by *Fusarium lycopersici* that in the earlier stages of the disease, when browning of the vascular strands was seen, there was upward transport of phosphate to the leaves from the roots. Hence this apparently normal activity of an infected plant in the earlier stages of toxæmia—normal in those regions of the leaves which are not yet affected by the lethal concentration of the toxins—could possibly be visualised in toxic wilting as the pure toxins are known to act specifically on certain host tissues (Gäumann, 1951), leaving other tissues unaffected. Moreover, the period of plant life following symptom appearance before complete death of these plants, which was seen to vary from 12 to 260 hours, depending on the age of the host at the time of infection, could be interpreted only on the basis of toxic wilting, as it is well known that any interruption in the supply of toxin could bring the plant to normal life and in fact, Varadarajan (1953) has demonstrated that Fe/Mn amendments to soils infected with *F. vasinfectum* could bring the plants to normal conditions even though the vein clearing symptom had made its appearance on the infected plants. The exact mechanism of resistance offered by Fe/Mn treated plants is, however, not clear.

The resistant strains of cotton plants (*Gossypium hirsutum*) were resistant to the wilt disease caused by *F. vasinfectum* in spite of their being infected by the pathogen under field conditions of pathogenicity. A study of the ascorbic acid and carbohydrate metabolisms of these infected plants indicated that it was a case of metabolic resistance to the disease (Kalyanasundaram, 1953). Such cases of metabolic resistance have been reported by Davis and Dimond (1952) in the case of tomato plants infected by *F. lycopersici*, brought about by treatments with chemotherapeutants. These instances of plant infection not culminating in the disease itself could not but happen in toxic wilting. Moreover, Gäumann and Naef-Roth (1953), working with the wilt toxin lycomarasmin on cut shoots of tomato, have shown that maximum toxic damage was seen in winter months and not in summer months. This they ascribed to the greater ascorbic acid synthesis of these shoots during summer months with greater light period.

Most of the work done in this laboratory has been on the wilt of cotton caused by *F. vasinfectum*, although wilt of tomato produced by *F. lycopersici* has been investigated widely in Europe (Plattner and Clauson-Kass, 1945; Gäumann *et al.*, 1952) and America (Gottlieb, 1944; Scheffer and Walker, 1953; Dimond, Waggoner and Dimond, 1954). All the same, both these pathogens are known to produce fusaric acid as one of their toxins and evidence of *in vitro* and *in vivo* studies presented here based on this toxin could be taken to explain the mechanism of wilting caused by these vascular Fusaria in general, unlike the studies on the wilt toxin lycomarasmin alone,

since it is highly specific to the causal organism *F. lycopersici*, and not known to be produced by any other species of *Fusarium*.

The *in vivo* symptoms reported earlier (Satyanarayana and Kalyanasundaram, 1952; Kalyanasundaram, 1954) could be brought about by the pure toxin fusaric acid, a 5*n*-Butylpyridincarbonic acid, with the empirical formula $C_{10}H_{13}O_2N$ and molecu ar weight of 179, isolated from *F. lycopersici*, *F. vasinfectum*, *F. hetero porum* and *Gibberella fujikuroi* (Gäumann *et al.*, 1952). The spectrum of action of fusaric acid is as unspecific as the spectrum of the causal organisms which form this toxin (Gäumann *et al.*, 1952; Kalyanasundaram, 1953). This toxin can cause damage to host plants of the families *Gramineæ*, *Papilionaceæ*, *Solanaceæ* and *Malvaceæ*. It is biologically interesting to note that the toxins of pathogenic fungi can kill different host plants, which the organisms are unable to attack under field conditions of pathogenicity. But, within this framework, considerable quantitative and qualitative differences are noticed. Cotton plants are hundred times more sensitive to fusaric acid than maize, twenty times more so than paddy and fifteen times more sensitive than tomato. This differing sensitivity of these hosts viewed in the light of differences in the synthesising capacity of the pure toxin by the three species of *Fusarium* under *in vitro* conditions—*F. moniliforme* (*Gibberella fujikuroi*) produces the highest concentration of fusaric acid in synthetic culture media followed by *F. lycopersici* and *F. vasinfectum*—naturally poses the question, " Is this a case of physiological specialisation in the genus *Fusarium* to parasitic adaptation ?"

Another factor affecting the biological activity of fusaric acid seems to be the pH of the substrate. A quantity of toxin which in the acid region (pH 4·3) caused stem necrosis damaged only the leaves in the alkaline region (pH 7·2). To cause stem necrosis in the alkaline range, the quantity and the concentration of the pure toxin have to be increased. This behaviour of the toxin seems to be due to the dissociation of the toxin in the alkaline ranges.

The production of antibiotics is a character of most of the soil inhabiting fungi. That *F. vasinfectum* belongs to this group was indicated by Subramanian (1950). Fusaric acid, one of the toxins of *F. vasinfectum*, also possesses antibiotic potency (Gäumann *et al.*, 1952). This was further established in this laboratory as could be detected by the bioassay method (Kalyanasundaram, 1955). Following this method this toxin could be detected in the culture filtrates of *F. vasinfectum* and *F. moniliforme*, even in one week old cultures. The early appearance of this toxin was mainly conditioned by the C/N ratio of the medium (Kalyanasundaram, unpublished). It is

necessary to refer here to the contention of Scheffer and Walker (1953) that lycomarasmin, one of the toxins of *F. lycopersici*, is produced in culture media only after two months as a product of lysis, and in almost all cases of wilt under *in vivo* conditions the plants hardly lived for 14 days after infection, and that within this period lycomarasmin could not be produced inside the infected plants. But it must be borne in mind that lycomarasmin is not the only toxin produced by *F. lycopersici* and the second toxin, fusaric acid, could be detected in culture media even as early as four days (Kalyanasundaram, unpublished) using the bioassay technique. Hence it is logical to assume that fusaric acid could be formed within the host in a short period of 14 days, within which period, however, lycomarasmin could not possibly be formed. Once the plant tissue is affected by the early formed fusaric acid, conditions may become conducive for the production of the lytic product, lycomarasmin. It is needless to stress once again that the phytotoxin lycomarasmin is highly specific to the causal organism *F. lycopersici*, unlike fusaric acid which is produced by many pathogenic species of *Fusarium*.

With the knowledge that phytotoxins produced by plant pathogenic fungi have also antibiotic property, we have to take into consideration the possible role of these antibiotics in the rhizosphere and root surface of host plants, especially in the light of recent evidence of gliotoxin production by *Trichoderma viride* in unsterilised soil (Wright, 1952) and stability of antibiotics in different types of soil (Jefferys, 1952).

The stimulation of the microflora in the rhizosphere region seems to be mainly due to the rich organic source of food by way of root secretions and sloughed-off materials. In fact, very recent work of Katznelson, Rouatt and Payne (1954) has shown the presence of amino acids in root secretions. Recent work of Kalyanasundaram (unpublished) has indicated that the pathogen, *F. vasinfectum*, produced considerable amounts of the toxin fusaric acid in sterilised soil amended with oats or green leaf choppings. It was demonstrated earlier by Agnihothrudu (1954) that *F. vasinfectum* was present in the rhizosphere and root surface of susceptible cotton plants far in advance of infection and symptom appearance. Naturally it leads us to hypothesise, that this fungus may produce considerable amounts of this toxin in the rhizosphere and root surface of susceptible host plants using the phenomenally rich food in those regions.

Whether or not this toxin which may be produced in the rhizosphere and root surface, can be taken up by the plant vascular system, as phytotoxins are known to be taken up by the plants from the soil (Brian, Wright, Stubbs and Way, 1951), it stands to reason to assume that the amount of

toxin produced can at least play a vital part in determining the resistance and susceptibility of the hosts as it is not clear whether toxin production is a prerequisite to infection or infection precedes toxin production, or both are simultaneous acts.

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SOME ASPECTS OF VARIATION AND VARIABILITY IN FUSARIA WITH REFERENCE TO THE TAXONOMY OF THE GENUS*

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The taxonomic division of a genus into species delimited by morphological characteristics is often difficult in those fungi in which fruiting bodies that are produced as a result of fusion of opposite sexes are not known to occur. This difficulty becomes all the greater in genera that show marked variation and variability in growth characters and in sporulation; species of such genera often tend to jump the limits of morphological boundary which is set to recognize the different specific forms.

The classification of the genus *Fusarium* has been both difficult and subject to inconsistencies. The tremendous variation within the species has posed the question of whether or not certain morphological characters, *viz.*, spore size and ability to produce different types of spores, can be considered as stable criteria for distinguishing species. Although the genus itself was established in 1809 by Link, it was not until 1935 that a system of classification was proposed for Fusaria (Wollenweber and Reinking, 1935). In this, as traditionally in other genera of fungi, species were separated primarily on the basis of their morphological characters, the size and shape of the various spore forms produced on standard media were taken as the chief criteria.

However, the system of classification proposed by Wollenweber and Reinking (1935) enjoyed wide popularity only for five years. In 1940 and in subsequent years, a group of American workers, criticizing Wollenweber's classification, pointed out that in this system morphologically homogeneous complexes had been divided into species that were differentiated on certain unstable characters which varied tremendously under different conditions (Snyder and Hansen, 1940, 1941, 1945; Oswald, 1949). Many of the earlier reports were also cited in evidence (Hansford, 1926; Brown, 1928; Mitter, 1929). Schreuder (1951) raised objection to the classification of Wollenweber and Reinking (1935) because in this, subtle mycological criteria used lead to the differentiation of a multiplicity of forms without phytopathological significance.

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Snyder and Hansen (1940, 1941, 1945) lumped together many of the closely related species recognized by Wollenweber and Reinking (1935) into a single species which, in their opinion, comprised these morphologically homogeneous complex of individuals; the 65 species described by Wollenweber and Reinking (1935) were reduced to nine species. Snyder and Hansen proposed a trinomial system of nomenclature in which species were divided into formæ, a forma name being given on the basis of physiological characters, *viz.*, differential pathogenicity and host specificity. Very recently, these authors have introduced the concept of horticultural variety for the recognition of subspecific forms; many of the intraspecific forms of *Fusarium* which show sufficiently recognizable differences would come under the category of ' horticultural varieties ' (Snyder and Hansen, 1954).

Many of the recent workers have been following Snyder and Hansen's nomenclature, although it may be mentioned that the trinomial system proposed by these two authors falls far short of its objective. It is becoming increasingly evident that pathogenicity in a species is as variable as morphological characters (Eide, 1935; Ullstrup, 1935; Borlaug, 1945; Venkata Ram, 1954). Some of the workers (Schreuder, 1951; Gordon, 1952), who have followed a somewhat middle course between the two systems of classification in *Fusarium* nomenclature, have accepted the resolution of species belonging to the sections Elegans and Martiella proposed by Snyder and Hansen. Schreuder (1951) states that for species belonging to the sections Discolor and Roseum which are readily distinguishable, the older names employed by Wollenweber and Reinking (1935) should be retained.

Miller (1941, 1946 *a*, 1946 *b*) in Canada published a number of papers in which he stated that Fusaria occur in nature as ' wild types ' which are characterized by abundant aerial mycelium with a few one-celled conidia. Cultivation of the ' wild type ' on artificial media invariably resulted in mutants being formed that sporulated profusely and which had lost considerably in aerial growth. He concluded that the size and shape of spores produced in culture could not be regarded as proper criteria in classification. Instead, Miller proposed that the morphological characteristics of the ' wild type ', as found in nature, be taken as criteria for the taxonomic division of Fusaria into species. According to this author the ' wild type ' showed a remarkable consistency in retaining its morphological characteristics.

But do all Fusaria occur in nature as ' wild types ' as defined by Miller (1945, 1946 *a*) ? Work of Subramanian (1951) has shown that this is not the case. Many of the Fusaria occur in nature as multiple forms of ' clones ', termed as ' natural clones ', which differ remarkably in many of the morphological characters as aerial growth and sporulation, thus supporting the

previous observations of Snyder *et al.* (1949). I have myself isolated numerous cultures of Fusaria from Southern Indian soils and I have found considerable variation in the type of 'clone' that appeared in the 'dilution plates', the variation was both interspecific as well as intraspecific.

None of the systems of classification proposed so far are without limitation and much is yet to be achieved in the taxonomy of Fusaria. Some of the aspects of variation and variability in Fusaria, how it affects classification of the genus, and future lines of approach to the problem will now be considered. It may be mentioned here, that in the classification of this genus it is extremely important to study variability of such of those characters that are considered as chief morphological features of the species.

Credit goes to Brown (1925, 1926) and Leonian (1929, 1932) for much of the early work on dissociation and variation in culture in the genus *Fusarium*. Brown and Horne (1924) recorded six isolates of Fusaria from diseased apples in nature which gave an assemblage of forms in culture; members of this assemblage derived from each isolate interdigitated with those derived from the others. From these results the authors concluded that the six original isolates were derived from a single parent. These early findings by Brown and his associates have two important bearings: (1) that a study of isolates in culture for their limits of variability is essential in determining species relationship, and (2) in nature, one species of *Fusarium* may occur as different clones, the morphological differences of which might provide a *prima facie* case for their separation into distinct species.

Variation in the composition of the food material and its concentration in the substratum has been found to influence mycelial type, and size and shape of spores (Brown, 1925; Brown and Horne, 1926). Saltations occur very frequently in the genus *Fusarium*, the frequency of this saltation has been found to be very high in certain media and in older cultures (Brown, 1926). Sometimes, distinct sectors are formed in the media although, mostly the new strains occur in isolated patches. These new strains sometimes remain different from the parent types, whilst in some cases they revert back to the original type (Leonian, 1929).

In *Fusarium solani* all the variants and progenies from a single individual were studied (Prasad, 1949) and it was found that striking differences existed between individuals in colony appearance, rate of growth, colour, and spore size. However, since all the variants were derived from one individual, it was concluded that all variations in cultural characters displayed would come under the normal range of variability in the fungus.

Numerous references could be cited pertaining to mutation in Fusaria, but perhaps none of such far-reaching importance as the work of Miller already referred to. This report (Miller, 1946 *a*) claimed that the freely sporulating cultures of Fusaria that produced sporodochia were only mutants formed as a result of cultural degradation in artificial media, and as such these did not occur in nature. It may be that the sporodochial form is produced from the ' wild types ' as a result of mutation in culture as shown in the case of *Fusarium lini* Bolley (Venkata Ram, 1955), but the possibility of this phenomenon occurring in nature cannot be ruled out. Indeed, Subramanian (1951) found that Fusaria do occur in nature in the sporodochial form.

Variants of Fusaria can occur by hyphal anastomosis of two or more isolates (Buxton, 1954). This phenomenon resulting in heterocaryosis, has been found to be quite common in the Fungi Imperfecti (Hansen, 1938, 1942; Jinks, 1952). Buxton (1954) showed that the cells of *Fusarium oxysporum* f. *gladioli* (Snyder and Hansen) were uninucleate throughout the mycelium excepting in the apical cells wherein 1 to 15 nuclei were found, an average of 7 per cell was recorded in the examination of 50 apical cells. Hyphal anastomosis of 2 apical cells of genetically different isolates, each containing homocaryons could result in many combinations of heterocaryons ultimately leading to numerous variants being produced. Mutation of one or more of these nuclei, not difficult to visualize, would result in a heterocaryotic condition. It is also noteworthy to mention here that at least two factors operate against the possibility of the uninucleate condition being continuous or permanent in fungi. These two factors, mitosis and anastomosis, operating singly or together, are able to produce two or more nuclei per cell (Hansen, 1942). Perhaps these findings explain the earlier results of Dickinson (1932) who, although finding hyphal anastomosis in *Fusarium* species, concluded that heterocaryosis could not be the reason for variation because of the uninucleate condition of the cells.

Many of the Fusaria are known to produce colour when grown on suitable substratum and this character has been taken for diagnostic purposes in species determination (Wollenweber and Reinking, 1935). Thus, a violet colour in the substratum is a strong indication of the Fusaria belonging to the section Elegans. That a single species can produce two colours, red and violet, in the same substratum has been recently demonstrated (Venkata Ram, 1953). Variants of *Fusarium lini* Bolley, grown on nutrient media containing different carbon sources and then transferred on to potato-sucrose media, produced colour in many shades of violet and red which were retained in subsequent cultural generations.

Mutants produced from a species differing in morphological characters differ in virulence and pathogenicity (Eide, 1935; Ullstrup, 1935; Miller 1945; Oswald, 1949); all these authors have demonstrated cultural variants to be less pathogenic than the parental type. In nature, a single species of *Fusarium* may have numerous pathogenic and non-pathogenic forms and other forms which are transitional between the most virulent and avirulent isolates (Borlaug, 1945). I have also found that many of the isolates of *Fusarium vasinfectum*, *F. oxysporum* and *F. solani* were capable of causing disease reaction in two hosts, cotton and red gram (Venkata Ram, 1954).

Alcohol production by isolates of *Fusarium lini* has been shown to vary considerably, pathogenic cultures were found to produce considerably more alcohol than did the non-pathogenic or weakly pathogenic isolates (Letcher and Williaman, 1926). Studies on the utilization of carbon and nitrogen *in vitro* have shown that Fusaria manifest considerable variability in their metabolism of these two elements (Pai, 1953; Venkata Ram and Gustafson, unpublished). One of the Russian workers (Fedotova, 1935) has shown that ability to utilize organic and inorganic sources of nitrogen is correlated with the pathogenic potentiality of the species. Fusaria also differ markedly in their ability to utilize pure cellulose, added to the medium in the form of filter paper, as a source of carbon. Recent work has shown that, whilst *Fusarium lateritium* and *F. sambucinum* completely dissolved the filter paper, *F. conglutinans* broke down the cellulose to a much lesser extent and *F. caucasicum* not at all, under identical conditions (Venkata Ram, unpublished).

Comparisons of the trace element nutrition of *F. vasinfectum*, *F. udum* and *F. moniliforme* showed that these are physiologically alike (Yogeswari, 1948). On the other hand, different isolates of *F. lini* Bolley responded quite differently to the presence of zinc and manganese in the culture medium (Venkata Ram and Gustafson, unpublished).

The response of Fusaria to organic dyes and growth-inhibiting substances was first studied extensively by Leonian (1929) who indicated that such tests might be invaluable in the identification of *Fusarium* isolates which showed considerable variability in growth and in morphological characters. Determination of a number of physiological reactions of the Fusaria belonging to the section Elegans, causing wilt of tomato, cabbage and muskmelon, showed that all the three behaved similarly in utilizing different carbon and nitrogen sources, but differed considerably in their response to various organic dyes and growth-inhibiting substances (Moore and Chupp, 1952). On this basis, the cabbage wilt Fusaria were distinctly separate from the other two, an observation supported by Armstrong and Armstrong (1952) who

upheld that the specific status of the cabbage wilt organism be retained as *Fusarium conglutinans* as recognized by Wollenweber and Reinking (1935) and not as a form of *F. oxysporum*, i.e., *F. oxysporum* f. *conglutinans*. Armstrong and Armstrong (1952) also stated that variants within the species of *F. conglutinans*, causing wilt of radish and stock, be recognized as physiologic races of *F. conglutinans*.

Fusaria of the sections Elegans and Martiella are known to produce toxic metabolites in culture which cause disease reactions in their hosts of choice (Gäumann, 1951). Some of these toxins are specific both to the host as well as to the pathogen (Winstead and Walker, 1954); a good example of this type of toxin is lycomarasmin which causes wilting in tomato and is produced by *F. bulbigenum* Cooke and Massee var. *lycopersici* (Brushi) Wr. et. Rg. The faculty to produce specific metabolites in culture will probably help in the identification of Fusaria, production of similar metabolic products by morphologically and genetically identical forms is visualized. Indeed, recent work in this laboratory has shown that culture filtrates of different species of *Fusarium* produce different reactions in cut shoots of cotton.

The metabolites of *Fusarium vasinfectum* and *F. bulbigenum* var. *lycopersici* have also been shown to stimulate profuse rooting in cut shoots of tomato and cotton; whereas, the metabolites of the former were found to be specific to cotton (Mostafa and Naim, 1948; Kalyanasundaram and Lakshminarayanan, 1952), the metabolites of *F. bulbigenum* var. *lycopersici* were non-specific (Mostafa and Naim, 1948). Recent investigations have shown that many of the other Fusaria of the section Elegans are also capable of stimulating rooting in cut shoots of specific and non-specific plants (Sadasivan and Subramanian, 1954); some of the Fusaria of the other sections have also manifested the ability to produce growth factors (Venkata Ram, unpublished). The presence of these growth-stimulating substances together with other vitamins and amino-acids present in the metabolites of many of the species is being investigated by the author in this laboratory, some of the results thus far obtained indicate that *Fusarium sambucinum* Fuck., *F. orthoceras* App. et Wr., *F. oxysporum* Schl., *F. conglutinans* Wr., *F. lini* Bolley, *F. bulbigenum* Cke. et Mass. var. *lycopersici* (Brushi) Wr. et Rg., *F. udum* Butler, *F. vasinfectum* Atk., *F. chlamydosporum* Wr. et Rg., *F. sporotrichioides* Sherbakoff, *F. moniliforme* Sheldon, *F. solani* (Mart.) App. et Wr., *F. buh ricum* and *F. caucasicum* synthezise appreciable quantities of thiamine and biotin which can be microbiologically assayed in the culture medium.

Application of the paper partition chromatography technique for the detection of sugars and amino acids is being made widely in biological sciences

and has been recently utilized in taxonomical studies (Bidwell *et al.*, 1952; Buzzati-Traverso and Rechnitzer, 1953); the use of this technique in studying species relationship in Fusaria suggested itself. However, preliminary work has shown that the differences in the composition of the fungal mycelium of different *Fusarium* species is not reflected in the pattern of free amino acids present. The amino acid pattern of the acid hydrolysate is still under investigation and details of this work will appear elsewhere.

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SOME BIOCHEMICAL ASPECTS OF VASCULAR WILTS*

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Toxin: General Considerations

In recent years considerable attention has been directed towards the study of microbial metabolic products by a number of workers in different countries (Raistrick, Arnstein and Cook in England, Gäumann and his school of workers in Switzerland, Dimond, Waggoner, Scheffer and Walker in the States) in view of their complicity in the production of many diseases by pathogens. They have been loosely called as toxins. The term toxin has been applied with so many different meanings in different contexts that it has become difficult to define it in precise terms. In therapeutics, the term is used to denote specific organic nitrogenous compounds produced by bacteria, in such diseases as tetanus, gas gangrene, diphtheria, etc. The term 'Wilting Toxin' was first applied by Gäumann (1950, 1951) to denote toxic metaoblites of fungal origin as enniatins, lycomarasmine, javanicin, etc., that were toxic to higher plants and claimed to be the primary cause of some or other of the syndrome of wilts. Vascular wilts were thus classified under toxigenic wilting. Toxins were claimed to be synthesized by the parasites (fungi or bacteria) as regular metabolic products in the course of their normal activity enter into the vascular system of the host plants and carried to the different parts of the plants wherein they initiate the various symptoms observed in the different organs. Dimond and Waggoner (1953 *b*) suggested a new term 'vivotoxin' defined as 'a substance produced in the infected host by the pathogen and/or its host which functions in the production of the disease but is not itself the initial inciting agent'. The definition of the toxin was limited to any compound produced by a micro-organism which is toxic to plants. Thus toxic metabolites isolated from culture filtrates were not regarded as vivotoxins unless their presence was detected in the naturally infected hosts and proved to be functional in the manifestation of the disease syndrome. In other words, they have suggested a modification of Koch's postulates in reference to toxins and consider these to be sufficient and necessary minimum conditions to establish vivotoxic complicity. The identification of the bacterial vivotoxin responsible for symptoms of tobacco wildfire

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disease provides an example of vivotoxicity by the modified Koch's postulates (Braun, 1950). While there are many practical limitations to a rigorous application of these postulates in many diseases of plants in view of the comparatively lower potencies of fungal toxins, unlike bacterial toxins, recent advances in analytical techniques as autoradiography (Wheeler, 1953), chromatography (Consden, Gordon and Martin, 1944; Dent, Stepka and Steward, 1947; Giri and Rao, 1952 *a*, *b*; Lakshminarayanan, 1954 *a*, *b*), etc., could be of considerable help in the *in vivo* detection of the toxic metabolites in different organs of plants.

Chemistry of the toxins.—The phytotoxins which have received considerable attention in recent years could be broadly classified into the three groups—peptides, quinones and organic acids. Among the peptides of phytotoxic importance are lycomarasmine (Plattner and Clauson-Kass, 1945 *a*, *b*; Gäumann, 1950); enniatins (Gäumann, Naef-Roth, Ettlinger, Plattner and Nager, 1947); lateritins I and II (Cook, Cox, Farmer and Lacey, 1947); sambucin and fructigenin (Lacey, 1950) isolated from different Fusaria. Javanicin and oxyjavanicin are among the important quinones of phytopathological interest (Arnstein, Cook and Lacey, 1946). Fusaric acid (Gäumann, Naef-Roth and Kobel, 1952 *a*, *b*) and vasinfuscarin (Gäumann, Stoll and Kern, 1953)—toxic metabolic products produced by many Fusaria, clavacin, an antibiotic produced by *Aspergillus clavatus* (Gäumann and Jaag, 1947) and patulin, produced by *Penicillium patulum* (Gäumann, Jaag and Braun, 1947; Miescher, 1950) are among the other important fungal metabolites which have received much attention. I shall confine my attention in the present paper to toxic metabolic products of Fusarial wilts of cotton and tomato. Lycomarasmine was first isolated by Plattner and Clauson-Kass in 1945 (*loc. cit.*) and later by Dimond and Waggoner (1953 *a*) from culture filtrates of *Fusarium bulbigenum* v. *lycopersici*, the causal organism of tomato wilt, grown in Richard's medium for 4 to 8 weeks. This was shown to be a simple peptide made up of the three amino acids L-hydroxyalanine, glycine and asparagine (Plattner and Clauson-Kass, 1945 *b*). The peptide was supposed to enter into chelation with $Fe^{\prime\prime\prime}$ at the -COOH group of the asparagine moiety (Dimond and Waggoner, 1953 *b*). The peptide is unstable, decomposes at 227–29° C. and forms a soluble barium salt. Aqueous solutions rapidly lose potency and ability to chelate with $Fe^{\prime\prime\prime}$. The iron complex was reported to be 10 times more toxic than the neat one when applied to cut shoots of tomato (Gäumann, 1951). The peptide gives positive ninhydrine reaction. Another product originally described as substance B (Plattner and Clauson-Kass, 1945 *a*) isolated from culture filtrates of *F. bulbigenum* v. *lycopersici* has been later identified as the

inactive form of lycomarasmine and might have arisen from the active form by the loss of a molecule of ammonia through hydrolysis. This is thermostable and has no action on tomato shoots.

Fusaric acid has been isolated from culture filtrates of different Fusaria and reported to be a pyridine carbonic acid (Gäumann *et al.*, 1952 *a*, *b*). It has no host specificity. Vasinfuscarin isolated from culture filtrates of *Fusarium bulbigenum* var. *lycopersici* was supposed to be responsible for vascular discoloration of tomato and provisionally identified as an enzymatic protein (Gäumann *et al.*, 1935).

Toxicological studies and symptomatology.—Similarity of disease symptoms to those produced in healthy hosts by toxic metabolic products is likely to be misleading since plants have a very limited way of expressing the symptoms unlike animals. A variety of unrelated chemical compounds could produce similar symptoms on the same host and in most cases such a similarity could at best be only suggestive of a similar *modus operandi* on host tissues. Dimond and Waggoner (1953 *b*) have elucidated this point with reference to the similarity of symptoms produced on tomato foliage by cucumber mosaic virus and many growth substances. In our laboratory Subramanian has observed that the symptoms produced on cut shoots of cotton treated to toxic concentrations of thiamine closely resemble those produced by dialysed culture filtrates of *Fusarium vasinfectum* (Subramanian, unpublished). In many cases a study of the biochemical disturbances in host plants would be not only essential but would also throw further corroborative evidence of disease manifestation.

Theories of Fusarium Wilts.—A rational understanding of the mechanism of *Fusarium* wilts is an essential prerequisite to formulating any programme of chemotherapeutic control of the wilts. The existing theories of *Fusarium* wilts could be broadly classified into the two groups, physical and chemical.

Physical mechanisms.—Increased permeability of the host cells has been reported by many workers in response to an uptake of toxic metabolic products of fungi (Gäumann and Jaag, 1947; Gäumann *et al.*, 1947; Gäumann, 1951). In our laboratory Lakshmanan has observed increased oxygen uptake in infected shoots of cotton over healthy ones (Lakshmanan, 1955). Since it is possible that any compound that can injure the host cells could produce increased permeability, it is doubtful that it could be a primary cause of wilt. Dimond and Waggoner (1953 *b*) have suggested that it is difficult to conclude if this is the cause or the effect. Besides an explanation based merely on increased permeability could hardly account for host and tissue specificities of the toxins. Gäumann (1951) reports that toxigenic

wilting is primarily accompanied by an injury to the semipermeability of the plasma membrane resulting in impairment of the osmotic prerequisite for turgor and thus takes place independently of the water balance. He differentiates this from physically induced wilting in that the latter is accompanied by a reduction in transpiration (cuticular and stomatal) in consequence of mechanical blockage of the intermicellar spaces of the cell walls.

Polysaccharides and other large molecules have been reported to cause wilting of tomato by many workers (Gäumann, 1951; Hodgson, Peterson and Riker, 1949; Scheffer and Walker, 1953). These have been recognized as metabolic products of fungal and bacterial growth (Dimond, Plumb, Stoddard and Horsfall, 1949; Dimond and Waggoner, 1953 *a*; Hodgson, Riker and Peterson, 1947). In vascular wilt diseases where the pathogen grows in the xylem vessels it has been suggested that the polysaccharides could be liberated in the vascular system in sufficient amounts to cause wilting. It may be pointed out that very large amounts of the polysaccharides are required for effective blockage of the capillaries, *e.g.*, 1700 mg. Inulin/Kg. fresh weight of tomato (Gäumann, 1951). Scheffer and Walker (1953) reported vessel plugging generally in stems and petioles of wilted leaves in inoculated tomato plants. Vascular browning was also reported in wilting leaves while the healthy ones showed no such browning. The fungus was isolated from the infected stems and petioles. Tomato cuttings of various ages exposed to replacement culture filtrates of the fungus in Czapek's medium were reported to show flagging of petioles and upper stems. The vessel plugging and browning factor was claimed to be thermostable (most of its activity was destroyed at 60° C./10 min.) and non-dialysable. The consensus of opinion is that lycomarasmine does not cause vascular plugging and browning and cannot be regarded as the principal wilting agent.

Chemical mechanisms.—Gothoskar, Scheffer, Walker and Stahmann (1953) and Winstead and Walker (1954) have recently attributed the bulk of the disease syndrome of tomato wilt to the pectin methyl esterase of the parasite. A purified preparation of the PME isolated from 3 to 6-day old bran cultures of *F. bulbigenum* v. *lycopersici* was found to reproduce many of the typical symptoms observed in natural wilts when applied to cut shoots of tomato. It was therefore suggested that the exo-cellular pectic enzymes of the parasite play a vital role in the production of vessel plugging and vascular browning. Dimond and Waggoner (1953 *b*) have suggested that a hydrolytic enzyme produced by the pathogen and attacking the lignin or other constituents of vessels or adjacent cells might yield coloured and viscous substances which could occlude the conductive elements of the stem and petiole. They have also indicated the possibility of secretion by the pathogen of an enzyme

which attacks the host in advance of mycelial growth as a plausible explanation of vivotoxic mechanism of *Fusarium* wilts. The possibility of Fusarial toxins acting as competitive or non-competitive inhibitors *in vivo* in the hosts has also been discussed by them though there has been no concrete evidence to that effect. Davis, Waggoner and Dimond (1953) have reported that certain orthophenols could produce vascular discoloration in tomato stems. Hydrolytic enzymes which liberate phenols from β-glucosides were also reported in cultures of different Fusaria and they have suggested that the carbohydrate fraction freed from the conjugated phenol could possibly function in the characteristic wilting mechanism.

Some argument against the theory of toxigenic wilting.—Presenting one of the major objections to the theory of toxigenic wilting Scheffer and Walker (1953) have observed that lycomarasmine is produced in quantity in liquid cultures only after 2 to 3 months while the fungus induces wilting in infected plants in 10 days and considered the toxin to be a lytic product. They have suggested that lycomarasmine production in cultures of *F. bulbigenum* v. *lycopersici* would be far less than that would be required to produce the wilt symptoms within the time the infected plants succumb to the disease. Dimond and Waggoner (1953 *b*) consider that this would be even on the lenient side in view of the fact that lycomarasmine in aqueous solution rapidly loses potency. We are not in agreement with their conclusion. It is quite probable that the susceptible host supplies ready organic precursors for the synthesis of the toxin while it is not so in synthetic media wherein a long lag period has to lapse before the necessary precursors, probably amino acids for the synthesis of the toxic peptide lycomarasmine, could be formed. It should be emphasized that the substrate composition and concentrations exert a vital influence on the synthesis of any microbial metabolic product, *e.g.*, penicillin production (Behrens, 1948) and the conditions offered by the susceptible host to the pathogen are not comparable to those in synthetic media.

In cut shoots, the toxin has been reported to produce a shock effect (Gäumann, 1951; Scheffer and Walker, 1953) which is not observed in the natural wilt. It may be pointed out that the conditions under which the toxin acts in the two cases are widely different. While it is a slow and steady secretion in the naturally diseased plants, the action is instantaneous in cut shoot treatments and the concentrations are also much higher.

In presenting further evidence against the toxin theory Scheffer and Walker (1953) have reported that different levels of $Fe^{\cdot\cdot}$ supplied to the host did not indicate any corresponding difference in its susceptibility to the action of lycomarasmine which has been shown to act only as the iron complex·

There is no indication in their work nor in Gäumann's as to the availability of free $Fe^{''}$ at the different levels for the chelation with the toxin lycomarasmine which seems to be a prerequisite for the activity of the toxin. Work on this aspect with resistant and susceptible varieties is underway and will be reported elsewhere.

Often, highly virulent strains were found to produce much less lycomarasmine than weakly pathogenic ones (Scheffer and Walker, 1953). Virulence will depend on the ability of the pathogen to metabolize the substrata offered by the host which as indicated earlier may not be comparable to a synthetic medium like Richard's. One major factor which Scheffer and Walker have not treated adequately is the effect of the toxin on resistant varieties of the plant. Gäumann (1951) has indicated that resistance of the host depends on its ability to check the generalization of the pathogen and that resistant cuttings were equally susceptible to the action of the toxin. In the resistant variety the infection remains restricted to the primary focus and the fungus fails to develop and more so produce a sufficient quantum of toxin. It is interesting to mention the grafting experiments Gäumann reported to establish the intake of the toxin by the transpirational stream. When scions of the susceptible variety of tomato, Bonny Best, were grafted on to resistant stocks, the fungus failed to develop and both the stocks and scions were healthy. On the other hand when the resistant scions were grafted to the susceptible stocks both showed wilt symptoms. Recently Davis (1954) reported some interesting intergeneric grafting experiments wherein susceptible tomato stocks were grafted to the scions of potato, tobacco, chilli pepper and tomato. Wilting and vascular discoloration were found in the scions even before symptoms could be observed in the stocks. The pathogen was isolated from the stocks but none from the scions except tomato. This offers further proof of the uptake of toxic metabolites in the transpirational stream. We are thus confronted with overwhelming evidence in support of the fact that toxic metabolites are produced in the host and play a vital role in the development of wilt symptoms.

Fusarium Wilt of Cotton by *F. vasinfectum*

Fusarium wilt of cotton has received considerable attention from many workers in this laboratory (Sadasivan and Subramanian, 1954). It has been reported that culture filtrates of *F. vasinfectum* contain an active toxic principle reproducing many of the typical symptoms accompanying the natural wilt of cotton (Kalyanasundaram and Lakshminarayanan, 1953). The active toxic principle was shown to be thermolabile and non-dialysable. The culture filtrates were found to contain at least two active principles, the

thermolabile toxic factor and a thermostable growth factor promoting profuse rooting in cut shoots even in dilutions. Mostafa and Naim (1948) have reported adventitious rooting in resistant cotton variety treated with crude culture filtrates of *F. vasinfectum* and claimed the factor to be thermolabile; no rooting was observed in the susceptible variety. It is probable that they were not dealing with the factors which we considered nor was the toxic effect of the unmetabolised culture ingredients considered. These two factors were reported by Satyanarayana (1953) to be biologically antagonistic in that the plants treated with the thermostable growth factor from germination did not develop wilt symptoms when grown in inoculated pots.

Nitrogen distribution in cotton plants.—One significant fact observed over a number of years in this laboratory between the susceptible and resistant varieties of cotton was that the latter invariably accumulated more total nitrogen than the former. Similar observations were also recorded in the root rot of cotton caused by *Macrophomina phaseoli* wherein the total nitrogen content of the plant was found to be directly proportional to resistance to the fungus (Chinnayya, 1951).

Detailed investigations by Satyanarayana (1953) revealed that the phenomenon of nitrogen accumulation was consistent irrespective of the nature or quantum of nitrogen supplied. The following varieties, Cambodia 2, Vijay, Madras Uganda I, Karunganni 2 and Malvi 9, in the decreasing order of resistance were investigated. Detailed studies on the nitrogen distribution in the different varieties in shoots, and roots grown in healthy, inoculated and wilt-sick soils were undertaken. Two interesting findings came out of these studies.

(1) Between the 6th and 12th day the susceptible shoots accumulated mostly non-protein nitrogen while the resistant ones did not. The latter seemed to synthesize the proteins far more rapidly than the former. In the susceptible varieties with the progress of infection from the 6th to 12th day, while the protein levels did not materially change NPN rapidly declined and the net effect of infection was the prevention of accumulation of NPN. This could be attributed to the powerful oxidative amino acid deaminase which I have observed (Lakshminarayanan, unpublished), in *F. vasinfectum*. The resistant Cambodia shoots presented a marked contrast to those of susceptible K 2 in that they accumulated the organic nitrogen mostly as proteins and this is probably associated with resistance and could account for the non-generalization of the parasite. The resistant plants showed no significant difference in the nitrogen distribution whether grown in healthy or inoculated soil. In studying the influence of the pathogen on the nitrogen

distribution in the hosts the NPN/PN ratios in the shoots on the 12th day presented marked contrast in susceptible and resistant plants. In the susceptible plants the diseased condition was accompanied by a reduction in the ratio while in the resistant the figures were steady (NPN/PN ratios on 12th day in shoots: K 2-Healthy: 6·87; Diseased: 2·58; Cambodia-Normal garden soil: 0·48, Inoculated soil: 0·42).

(2) The roots of the two varieties also presented similar striking contrasts. In the healthy K 2 the root protein nitrogen (PN) went down with the growth of the plant, from 6th to 12th day, *i.e.*, the seat of protein synthesis shifted from the root to the shoot. In the diseased state the PN increased in the roots. Cambodia did not reveal any significant change whether grown in healthy or inoculated soil.

It is relevant to point out that in the susceptible varieties the shoots possibly lose NPN, in the infected state, which go to form the toxic peptides responsible for the disease syndrome. This does not happen in the resistant varieties which rapidly achieve protein synthesis in shoots and do not have free NPN substrata for the enzymatic activity of the pathogen. In the resistant varieties the pathogen is not able to generalize since it is apparently unable to utilize the proteins. A suitable choice of a chemotherapeutant for Fusarial wilt control in cotton would probably be any agent that could accelerate protein synthesis in the susceptible shoots.

Chromatographic studies on free amino nitrogen constituents.—In view of the striking contrast presented by the susceptible varieties of cotton in the accumulation of organic nitrogenous constituents it was considered desirable to have a detailed picture of the free NPN constituents in the roots, shoots and leaves of the two varieties employing the techniques of paper disk chromatography and microchromatography developed by me (Lakshminarayanan, 1954 *a*, *b*). Extensive chromatographic studies on the distribution of α-amino constituents, positive to ninhydrine, in the two varieties of cotton K 2 and Cambodia revealed the interesting fact that in the resistant variety of cotton both shoots and roots, particularly the latter, contained significant amounts of cystine besides the other major constituents as glutamic and aspartic acids and other minor amino acids, while the susceptible ones did not reveal any cystine (details will be published elsewhere).

Role of iron and cystine chelation in toxigenic wilting of cotton.—The presence of cystine in the resistant variety and its absence in the susceptible one indicated the need to investigate if cystine was in any way related to resistance to wilt, both in presence and absence of ferric iron, particularly in view of the earlier reports that the antibiotic activity of clavacin was due

to its chelation to sulfhydryl group (Miescher, 1950; Cavallito and Bailey, 1944; Cavallito and Haskell, 1945; Geiger, 1947; Geiger and Conn, 1945). It is of particular interest to mention in this connection the work of Braun (1950) on the biochemical behaviour of tobacco wildfire toxin wherein he reported that the addition of l-methionine overcame the toxic effects of culture filtrates of *Pseudomonas tabaci* which failed to produce a chlorotic halo in tobacco leaves. The mechanism was claimed to be competitive inhibition. This was further confirmed by the fact that the methionine antagonist methionine sulfoxime produced halos indistinguishable from those produced by the toxin on tobacco leaves.

Our experiments gave the interesting result that the dialysed culture filtrate of *F. vasinfectum* was comparable to lycomarasmine in relation to $Fe^{\prime\prime\prime}$ which greatly increased the toxic symptoms as shrivelling, yellowing and vein-clearing at an optimum level of 10^{-3} M. when applied to cut shoots of K 2, 10 to 12-days old, grown in garden soil. Besides, Subramanian (1954, unpublished) has observed in our laboratory, that 8-quinolinol when added to sand cultures at an optimum level of 5 p.p.m. gave effective protection against wilt in inoculated series. Cut shoot experiments gave findings similar to those reported by Waggoner and Dimond (1953) for lycomarasmine. Cut shoots of K 2 pretreated with 8-quinolinol at the optimum level for 24 hours, gave effective protection against the toxic effects of culture filtrates of *F. vasinfectum*. These results suggest that the active toxic principle of *F. vasinfectum* responsible for the wilt syndrome of cotton acts in a manner analogous to lycomarasmine on tomato by forming a toxic chelate complex with $Fe^{\prime\prime\prime}$. Furthermore, elimination of iron from the culture medium (by the technique of purification used in this laboratory) resulted in very poor toxicity of the culture filtrate.

Cystine was not toxic at levels lower than 10^{-4} M. and gave effective protection to cut shoots when prechelated to $Fe^{\prime\prime\prime}$ in presence of the toxin. In the absence of iron, cystine produced no significant beneficial effects. Cystine also increased chlorophyllation at the subtoxic levels. Cut shoots pretreated with cystine at the optimum level for 24 hours were able to overcome the toxic effects of the culture filtrate. These results suggest the possibility that the cystine present in the resistant variety of cotton chelates with $Fe^{\prime\prime\prime}$ available in the host and renders it unavailable for chelation with the toxin which seems to be the prerequisite for toxigenic wilting. Further work on the mechanism of cystine liberation and its chelation is underway and is likely to throw some light on the mechanism of resistance in cotton.

Dimond and Waggoner (1953 *c*), however, were not able to obtain any beneficial effects in antidoting the ferrous or ferric lycomarasmine complex with cysteine, glutamic acid, glutamine or glutathione.

Role of enzyme systems of the parasite in pathological wilting.—While the foregoing discussion would reveal that there is considerable evidence of the *in vivo* action of the toxic metabolites of the pathogen in *Fuarium* wilts, the exact mechanism of their formation and action are not yet clearly understood, since their detection *in situ* has not been achieved in many cases. However, as early as 1944, Gottlieb reported conclusive evidence establishing the presence of the toxin in tracheal fluids of wilt-infected tomato plants by *F. bulbigenum* var. *lycopersici* and indicated that while the mycelium was present in the roots and the lower parts of the stem no fungal growth could be detected in the upper parts; in no case was there enough plugging to account for the wilting. In natural infections it is possible that the pathogen invading the host might secrete more vivotoxins than could be detected in static cultures and would account for the variety of symptoms which the live pathogen induces on the host. It is necessary to emphasize that in the natural infections two active life-cycles, *viz.*, that of the host and the pathogen, exist together and compete for the metabolic reserves, while in synthetic cultures such a dynamic phenomenon does not exist. The role of the endo- and exo-cellular enzymes of the parasite in the production of wilts has been discussed by me earlier (Lakshminarayanan, 1953). Our findings also indicate that many of the typical symptoms of cotton wilt as vein-clearing, dechlorophyllation, carbohydrate injury, etc., could be reproduced by commercial takadiastase; it is possible that the diastatic enzymes of *F. vasinfectum* could account for at least a portion of the disease syndrome. While a number of toxins, particularly of bacteria, could act directly as enzymes themselves (*e.g.*, lecithinase activity of *Cl. Welchi*—Gäumann, 1951) in animal metabolism, phytotoxins could be products of enzymatic activity of pathogenic fungi on susceptible host substrata and part of the disease syndrome could be the direct consequence of their tissue specificity. It is only a critical probe into the enzymatic complexes of the parasite and their activity *in situ* in the hosts that could give the exact mechanism of wilt diseases. Neither completely physical explanations as vessel plugging or increased permeability or chemical explanations based on *in vitro* toxicity experiments with culture filtrates under limited and artificial conditions could provide a rational explanation of the mechanism.

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HEAVY METAL REQUIREMENTS OF FUNGI*

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The fungi need certain metallic as well as non-metallic elements to supply their nutritional requirements. These are known as the essential nutrient elements. A biologically essential element is indispensable in the sense that, without it growth or any other vital activity of an organism is markedly impaired, or even impossible, and that it cannot entirely be replaced by any other element or compound. The amounts required vary with each element and every organism. Even strains of the same species differ widely in their growth requirements. In general, the non-metallic elements, which form the major portion of the structural composition of fungi, are, understandably, needed in considerable amounts. The metallic elements, however, are required in relatively small quantities. The heavy metals, in particular, are needed only in such minute traces that they have often been referred to as 'trace elements' or 'micro-nutrients'. In spite of the small amounts required, these are as essential in vital processes as are the major elements.

Studies designed to evaluate the biological essentiality of any element demand the use of chemically well-defined media. A single element only may be studied at a time, by omitting that alone from the otherwise complete medium. A medium absolutely free from the element under study is the ideal, but this is difficult to attain in practice. The experimental difficulties increase as the amount of element required becomes less.

Being needed only in small traces, the heavy metals are usually supplied in sufficient quantities to meet the requirements of most fungi, as impurities from the culture materials ordinarily used in the laboratory. The chemicals form a chief source of heavy metal impurities. Even the so-called 'chemically pure' substances are grossly contaminated from the biological point of view. A medium prepared with these chemicals needs rigorous purification before it can be successfully used to detect heavy metal essentialities. Equally important is the quality of glassware employed. Although quartzware is most preferable, 'Pyrex' or 'Hysil' brand has been generally used with success. The water to be used should be distilled over again in high quality glass stills. All glassware should be thoroughly cleaned with strong acid

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detergents, washed profusely with glass-distilled water, finally rinsed in twice-, or preferably, thrice-distilled water and dried. Careful handling of chemicals, discriminate use of glassware, avoiding dust as far as possible, etc., go a long way in the successful management of these highly critical experiments.

It has been the usual practice in studies with fungi, and other micro-organisms, to purify the entire basal medium as such rather than the several components separately. Success in essentiality studies with heavy metals largely depends on efficient methods of removing them from the media employed. No one method is useful to remove all heavy metal impurities at once. Moreover, the degree of purity to be attained depends on the amount of element required and the sensitivity of the organism studied.

The various chemical methods of purification (adsorption and chelation methods) recommended by several workers have been compared, trying certain combinations and modifications thereof, by some Australian workers recently (Donald, Passey and Swaby, 1952 *a*). They have also devised a method using a new adsorbing agent (Al_2O_3) for certain heavy metals. The original calcium carbonate method introduced by Steinberg (1919)—using which he, the pioneer in this field, has been able to demonstrate the essential nature of most of the trace elements that, surprisingly enough, still remain the only few investigated—, is found to be less efficient in the hands of recent workers who have, nevertheless, developed alternative methods. The scarcity of available, efficient techniques explains the limited number of elements investigated as regards their biological essentiality.

Most of our knowledge on heavy metal nutrition of fungi is confined to the fungus *Aspergillus niger* and the elements Fe, Zn, Cu, Mn and Mo. The essentiality of Fe and Zn to this organism has been claimed as early as 1869 by Raulin, although confirmed only much later by Steinberg (1919) who introduced and developed the fastidious techniques which such work calls for. Cu, Mn and Mo were subsequently added (Steinberg, 1934; 1935; 1937). Taking extraordinary care he was also able to indicate the essential nature of Ga in the normal growth of the same fungus (Steinberg, 1938). A few other fungi which have been shown to require Zn, Cu and Mn include *Rhizopus nigricans* and *Aspergillus flavus* (McHargue and Calfee, 1931), *Ceratostomella ulmi* (Ledeboer, 1934), *Trichophyton interdigitale* (Mosher, Saunders, Kingery and Williams, 1936) and *Phymatotrichum omnivorum* (Rogers, 1938). The stray claims on the essentiality of a few other elements, *viz.*, Ga (Steinberg, 1938), V (Bertrand, 1941), Sc (Steinberg, 1939), Cb and W (Lockwood, 1933) and B (Davis, Marloth and Bishop, 1928; Yogeswari,

1950), remain inconclusive, and most of them are based on an increased growth obtained on addition of the elements concerned.

The fungi, as a group, are highly responsive to external conditions and this property has been taken advantage of in bioassay of various substances such as vitamins, amino acids and mineral elements. These bioassay methods are based on Liebig's ' law of limiting factors'. Accurate quantitative estimations have been possible on account of the high specificity and stability of the test organisms chosen, only a few in practice as against the theoretically unlimited number. Various strains of *Aspergillus niger* have been used by different workers to estimate available quantities of heavy metals as well as other mineral nutrients, in soils (Hewitt and Hallas, 1951; Donald, Passey and Swaby, 1952 *b*; Dole and Welsh, 1952; earlier literature in Nicholas, 1950) and plant materials (Hewitt and Hallas, 1951; Nicholas and Fielding, 1951), the standard ' M ' strain being the most widely used.

This organism gives characteristic responses to specific heavy metal deficiencies. Growth and sporulation are the criteria assessed which increase in amount and colour intensity proportionately to increasing amounts of each of these elements until the normal state (a wrinkled mat and a complete, black spore cover) is reached. Based on this, standard growth series have been prepared, using the ' M ' strain, for Fe, Zn, Cu, Mn and Mo. The sensitivity of this strain to some of these heavy metals far exceeds that of critical chemical tests, particularly so in the case of Mo where, as low a level as 0·0001 μg./50 ml. culture solution could be detected (Nicholas, 1950).

In this laboratory, the basal medium used in heavy metal essentiality studies has been purified according to the Al_2O_3 method devised by the Australian workers, for studies with Fe, Zn, Cu and Mn, and according to the H_2S co-precipitation method, as advocated by Hewitt and Hallas (1951), for those with Mo. The biological test, using the ' M ' strain of *Aspergillus niger*, was carried out to assess the degree of purity attained. Considering the usual criteria, the purified medium was found to be sufficiently free from traces of Fe, Zn, Cu and Mo (Saraswathi-Devi, 1954). Later trials employing H_2S method (normally used to remove Mo) for removing Cu gave better results than Al_2O_3 method (Saraswathi-Devi, unpublished). Despite all precautions possible, the typical ' cauliflower ' growth (Donald, Passey and Swaby, 1952 *a*) of the standard strain in Mn-deficient cultures could not be obtained. In this connection, it is worthwhile to point out that this may only be an indication of the practical difficulties involved in such critical studies. Studies with another strain of the same fungus (M.U.B.L., 1) indicated the possibility of its greater sensitivity than the ' M ' strain towards

traces of Cu and Mo. The new strain, however, produced the typical 'cauliflower' growth, with no spores at all, in the Mn-deficient cultures, naturally revealing its larger need for this element than the standard strain (Saraswathi-Devi, 1954).

Ensuring thus a satisfactory degree of purity of the basal medium as well as other materials employed, the essentiality of heavy metals to certain plant pathogenic fungi was investigated. The indispensability of Fe and Zn for normal growth (expressed as dry weight yield of the fungus) of nine species of *Fusarium* (*F. vasinfectum*, *F. moniliforme*, *F. udum*, *F. scirpi*, *F. orthoceras*, *F. oxysporum*, *F. lini*, *F. poæ* and an unidentified *F.* sp.), mostly wilt pathogens, has been proved. In the absence of either of these elements from the medium supplied, growth in all species was greatly reduced as compared to that obtained in their presence, in some cases even to 3% of the maximum (Saraswathi-Devi, unpublished).

Fusarium vasinfectum, the cotton wilt pathogen, was then taken up for detailed investigation. Studying the effect of Zn, varying concentrations of this element (0–100 μg./25 ml. medium) were supplied to the purified medium amended sufficiently with constant amounts of Fe, Cu, Mn and Mo. From the very poor growth in the Zn-omitted cultures the organism steadily improved until it reached the sufficiency level, where normal growth was attained. Further increase in Zn supply appeared neither to increase nor decrease the amount of growth to any considerable extent. Sporulation, however, was seen to increase consistently up to the highest level tried (Saraswathi-Devi, unpublished).

In an attempt to study the influence of Zn on other physiological activities of *F. vasinfectum*, its ability to produce fusaric acid, one of the phytotoxins known to be produced by this organism, was assessed using the bioassay method developed in this laboratory (Kalyanasundaram, 1955). Assaying filtrates of 15 day-old cultures (supplied with various levels of Zn), it was found that a certain minimum was essential for any fusaric acid production. With the very low levels of Zn supplied no fusaric acid was produced, in spite of the organism having made a certain amount of growth, though very deficient. Only at a level where the fungus attained appreciable growth did this toxin become detectable. Peak toxin production was noted at a level higher than that required for normal growth. In contrast to the growth response of the organism, higher concentrations of Zn markedly reduced fusaric acid production, being completely inhibited at the highest level tried. Within this short range of Zn concentrations for toxin production, the rise from the minimum to the optimum level and the fall thereafter, seemed very rapid (Kalyanasundaram and Saraswathi-Devi, 1955).

It has been reported earlier that Zn amendments to wilt-sick soil controlled severity of cotton wilt to a great extent (Sulochana, 1952). Also, in view of increasing experimental evidence in support of the toxin theory of plant wilts, the afore-mentioned effect of Zn on toxin production by the cotton wilt pathogen, although still in preliminary stages of investigation, seems to be of considerable interest.

In early experiments with a few species of *Fusarium*, the omission of Cu, Mn or Mo did not appreciably decrease growth (Saraswathi-Devi, unpublished). That chances of certain vitamins (thiamin and biotin) replacing Fe or Zn in entirety, were also noted to be little, both in the case of *F. vasinfectum* (a fungus autotrophic with regard to vitamins) (Saraswathi-Devi, unpublished), and a strain of *Piricularia oryzæ* (the paddy-blast fungus) shown to be deficient for certain vitamins (Apparao, Saraswathi-Devi and Suryanarayanan, 1955).

Further studies on the heavy metal nutrition of these pathogenic soil fungi under varying cultural conditions are in progress. Considering the vital enzymic roles assigned to these trace elements, it is not probable that certain organisms can carry on normal growth and metabolism without some o the elements proved to be essential for others. It is most likely that the amounts required are so minute that much higher degrees of purity of materials used, than at present available, become desirable to study essentialities of these trace metals. Sufficient enthusiasm and curiosity on the part of investigators in the various fields of science may help development of highly refined techniques and biologically pure substances in the not distant future, when more uniformity in growth requirements of fungi, and other microorganisms in general, may well be expected.

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RESPIRATORY STUDIES ON VASCULAR WILTS*

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Beginning with the classical researches of Pfeffer (1900) aerobic respiration has come to be regarded as a primary factor for plant growth since it provides the main source of available energy and the principal raw material for plant physiological functions. Our main interest in the study of tissue respiration in cotton plants was that of correlating the respiratory status of host tissues with the degree of resistance or susceptibility to the pathogen *Fusarium vasinfectum* causing vascular wilt of cotton. Vascular occlusion either by fungal mycelia or by polysaccharides released into the system has been claimed by many workers to be the primary cause of fusariose wilts (Gäumann, 1951; Hodgson, Peterson and Riker, 1949). Increased permeability and a destruction of osmotic prerequisite for turgor have also been indicated by Gäumann (1951) to be the basis of toxigenic wilting. Since an injury to the host tissues in toxigenic wilting would be reflected in respiratory changes, it was considered desirable to make a detailed study of oxygen uptake and carbon dioxide liberation in host tissues of resistant and susceptible strains of cotton under different environmental conditions both in healthy and diseased states.

Three strains of cotton were chosen Karunganni 2 (*Gossypium arboreum*), Cambodia 2 and Madras Uganda 1 (strains of *G. hirsutum*) of which the latter two are resistant to the pathogen. Sections were taken with a cylinder microtome. It was found that stem sections of 3 mm. thickness gave more consistent and reproducible respiratory values than thinner ones. The gas exchange was measured in Barcroft manometers by the direct method (Dixon, 1951).

Our own observations indicate that in the 7-, 14- and 21-day plants of the healthy susceptible cotton the respiratory intensity of the stem tissues was in the decreasing order of 7-day > 21-day > 14-day plants. However, the difference in the respiratory intensity between the 21- and 14-day plants was negligible; but was considerably less than that of the 7-day series (Text-Fig. 1). Similar observations were recorded by Hover and Gustafson (1927) working on leaves of corn, sorghum, oats and wheat. On the other hand

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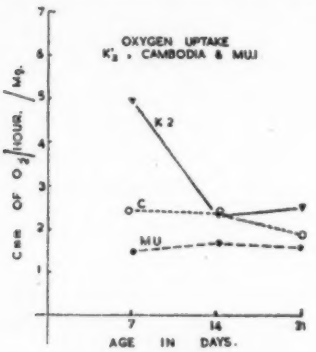
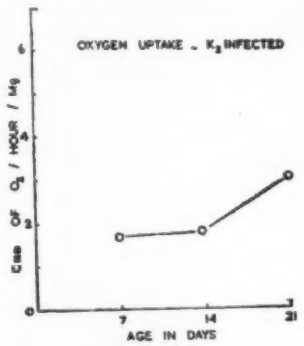
in the case of resistant strains of cotton (Cambodia and M.U.I.) no significant differences were observed between the three different age levels studied. Generally comparing the varieties, the susceptible one showed a higher rate of oxygen uptake than both the resistant strains (Text-Fig. 1).

A study of the respiratory changes in susceptible cotton (var. K 2) during pathogenesis has shown that there is a gradual increase in the intensities of respiration with age of the plants grown in sterilized soil containing 5 per cent. inoculum of *Fusarium vasinfectum*, the increase between the 14- and 21-day plants being marked (Text-Fig. 2). It is interesting to note that despite the external manifestation of the earliest visual symptom of the disease, *viz.*, 'vein clearing' on the 14-day plant it was only in the 21-day plant, when both the cotyledonary and the first pair of leaves showed vein clearing that there was nearly a hundred per cent. increase in the intensity of respiration over the initial rate (Text-Fig. 2). In available literature on this subject



TEXT-FIG. 1.



TEXT-FIG. 2.

(Gäumann, 1951) increased respiration has been recorded following fungal infection of host tissues. However, in another type of pathological disease syndrome produced by different plant viruses in different hosts both increased and decreased respiration have been recorded (Bawden, 1950). The mechanism by which these respiratory changes are brought about is still obscure. I need hardly mention that more work needs to be done on the factors that regulate the rate of respiration particularly the respiratory enzymes in the pathological state and should be correlated with the actual changes in a given tissue. The effects of toxins on tissue respiration have recently been reviewed by Allen (1953). The view that chemical substances produced by the parasite are the principal cause of pathological wilting of plants implies the production by the parasite of substances capable of producing severe metabolic changes in the host. Hellinga (1940) using thin slices of potato tubers found

that oxygen uptake and carbon dioxide production were increased by extracts of the mycelium of *Gibberella saubinettii*, the causal organism of soft-rot of potatoes. Smaller increases were produced by culture filtrates and by extracts of infected tubers. Denny (1924) has reported increased respiration of lemon fruits exposed to small concentrations of ethylene, which was identified by Young, Pratt and Baile (1951) as a volatile product of the rot fungus *Penicillium digitatum*. A pathological increase in carbon dioxide production was observed by Gäumann (1951) in tomato plants subjected to the action of lycomarasmin, the wilt inducing toxin produced by *Fusarium bulbigenum* v. *lycopersici*, the pathogenic agent of tomato wilt. He has also observed a coagulating effect on the plasma and a disruption of the semi-permeability of the plasma membrane. Growth substances are also known to influence tissue respiration in plants in a similar manner. Many investigators have shown that growth substances can increase the exogenous oxygen uptake of plants and tissues (Commoner and Thimann, 1941; Berger, Smith and Avery, 1946; Kelly and Avery, 1949; Anker, 1951). Growth substances have also been known to increase the endogenous oxygen uptake of tissues (Anker, 1953). This aspect of growth substance action is explained by a sensitizing effect on inner lipophilic protoplasmic films which presumably separate the enzymes and their endogenous substrates. A similar explanation is given by Stenlid (1949) to explain the stimulation of 2, 4, dinitrophenol on the respiration of wheat roots. He suggested a destruction of some cell structures which leads to increased contacts between some respiratory substrate and an enzyme system not working with full efficiency.

From the foregoing discussion it may be concluded that both growth substances and toxins act on the protoplasmic membrane resulting in an increase in the intensity of respiration though the ultimate effects on the host are different; one resulting in growth and the other in toxæmia.

The problem now confronting us is one where intensive work is to be undertaken on the respiratory rates of many host tissues in the presence of neat metabolic products of Fusaria producing not only toxæmia but also the stimulatory effect of rooting on cut shoots by dialysed heat treated culture filtrates (Kalyanasundaram and Lakshminarayanan, 1953).

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